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Oscillating CD8+ T Cell Effector Functions after Antigen Recognition in the Liver

Masanori Isogawa, Yoshihiro Furuichi, and Francis V. Chisari*
Department of Molecular and Experimental Medicine
The Scripps Research Institute
La Jolla, California 92037

Summary

When hepatitis B virus (HBV)-specific CD8+ cytotoxic T lymphocytes (CTLs) are adoptively transferred into HBV transgenic mice, they enter the liver, recognize antigen, secrete interferon γ (IFNγ), inhibit viral replication, and kill their target cells, causing hepatitis. In the current study, we examined the impact of antigen recognition on the evolution of the activation phenotype, antiviral effector functions, expansion and contraction kinetics, and compartmentalization of the transferred CTLs. The results reveal that noncytolytic and cytolytic effector functions and expansion-contraction kinetics of the CTLs are regulated asynchronously and in an oscillatory manner as a consequence of antigen recognition in the liver and in association with PD-1 upregulation. We suggest that such oscillations maximize viral clearance and minimize tissue injury during HBV infection and that poor coordination of these events could lead to viral persistence and chronic liver disease.

Introduction

Although it is widely accepted that a vigorous virus-specific CD8+ T cell response is required to control most viral infections, the extent to which the cytolytic and noncytolytic functions of those T cells must be coordinated for viral clearance to occur is not well defined. Upon TCR engagement with peptide-MHC class I complexes, CD8+ T cells massively expand (Flynn et al., 1998; Murali-Krishna et al., 1998) and express multiple effector functions, including cytotoxicity mediated by perforin-granzyme and Fas-FasL pathways (Berke, 1995; Harty et al., 2000), and cytokine production such as IFNγ and TNFα (Harty et al., 2000; Slifka et al., 1999). These two effector functions differ in several ways. First, cytotoxicity is limited to target cells that are in direct contact with CD8+ T cells, whereas cytokine release can have both local and systemic consequences (Slifka and Whitton, 2000). Second, cytotoxicity relies on the presence of stored molecules in lytic granules or FasL expression on their surface (Barry and Bleackley, 2002), whereas secretion of antiviral cytokines requires de novo synthesis of effector molecules (Slifka et al., 1999) upon encountering antigen. Third, most antigen-specific CD8+ T cells constitutively express perforin and granzyme B (grB), but not cytokines ex vivo, at the peak of the antiviral immune response (Slifka et al., 1999; Wherry et al., 2003b). Although these findings suggest that regulation of these two effector functions is fundamentally different, the relative importance and kinetics of CD8+ T cell-mediated IFNγ production and cytolytic activity in controlling virus infection have remained elusive. For example, although the rapid kinetics of CD8+ T cell cytokine induction by antigen has been well characterized in vitro (Lalvani et al., 1997; Slifka et al., 1999), the relationship between the kinetics of CD8+ T cell expansion and contraction, cytokine and activation marker expression, cytolytic effector function, viral clearance, and disease pathogenesis is not completely understood.

We have previously studied the impact of antigen recognition by adoptively transferred HBV-specific CD8+ CTLs on HBV replication and liver disease pathogenesis (Ando et al., 1993; Guidotti et al., 1996). In those studies, we showed that antigen recognition in the liver triggers the release of antiviral cytokines that inhibit viral replication noncytopathically (Guidotti et al., 1996) and that it activates the cytolytic potential of these T cells, resulting in a necroinflammatory liver disease (Ando et al., 1993; Guidotti et al., 1996) with kinetics that are delayed relative to the antiviral effect. In keeping with these observations, we have recently shown that the onset of viral clearance in HBV-infected chimpanzees is tightly associated with the appearance of IFNγ-producing virus-specific T cells as well as IFNγ mRNA in the liver and that it is well underway long before the peak of liver disease (Guidotti et al., 1999; Thimme et al., 2003; Wieland et al., 2004). In the current study, we examined the impact of antigen recognition on the adoptively transferred CTLs themselves, focusing on their activation profile, effector functions, and expansion and contraction kinetics in the liver and spleen of HBV transgenic recipients, and we related these changes to the dynamics of viral replication, viral gene expression, and liver disease. The results demonstrate that target cells, in this case hepatocytes, talk back to the CTLs, resulting in a sequential and oscillatory response pattern in the CTLs that could have a significant impact on the outcome of the host-virus interaction during HBV infection.

Results

Induction and Characterization of HBsAg-Specific CD8+ T Cell Response in Donor Spleen Cells

A group of 50 B6D2 mice were injected intramuscularly with 100 μg of plasmid encoding the HBV middle envelope protein (pCMVS2.S) in order to prime a CD8+ T cell response to HBsAg. 3–4 weeks later, they were intravenously infected with 1×10^7 pfu of recombinant vaccinia virus that expresses the HBV major envelope protein (vHBs4). 14 days after the booster immunization, the hierarchy of HBsAg-specific CD8+ T cells in the spleen was analyzed by intracellular cytokine staining (ICS) for IFNγ after 5 hr of in vitro stimulation with the known HBsAg-derived, H-2^d-, and H-2^b-restricted CTL epitopes ENV28, ENV190, ENV208, ENV362, and ENV364.

*Correspondence: fchisari@scripps.edu

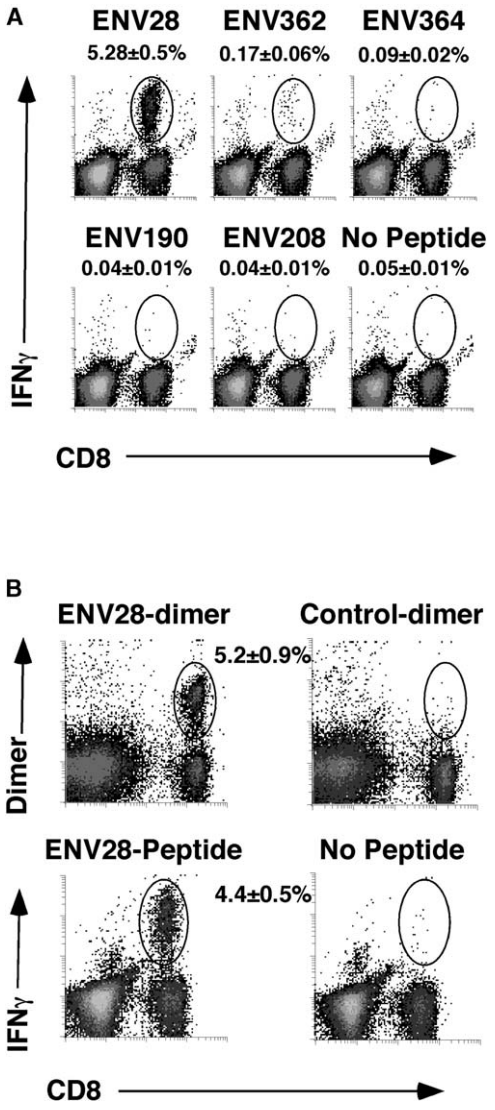

Figure 1. Characterization of HBsAg-Specific CD8+ T Cell Response Induced by Intramuscular DNA Prime-Vaccinia Boost Immunization

Mice were first primed by intramuscular injection of 100 μg of pCMV-S2.S DNA that expressed HBV middle envelope protein. 2–3 weeks later, they were infected with 1×10^7 pfu of HBs4 that expresses HBV major envelope protein. 2 weeks later, mice were sacrificed to obtain spleen cells. The hierarchy of CD8+ T cells to known H-2b- and H-2d-restricted CTL epitopes was examined by using ICS for IFNγ (A). The frequency of ENV28-CD8+ T cells was compared by using dimer staining and ICS (B).

As shown in Figure 1A, the ENV28-specific CD8+ T cell response was strongly immunodominant, representing more than 95% of the response to the tested peptides. Therefore, the ENV28-specific CD8+ T cell response was monitored in the following experiments.

We then compared the number of ENV28-specific CD8+ T cells that produced IFNγ in response to the ENV28 peptide with the number of CD8+ T cells that could be detected with the corresponding ENV28-dimer. As shown in Figure 1B, 5.2% ± 0.9% of total spleen cells (41% ± 7.0% of CD8+ T cells) express specific TCRs that recognize ENV28-L^d complex detected by ENV28-dimer, whereas 4.4% ± 0.5% of total spleen cells (38% ± 4.31% of CD8+ T cells) produce IFNγ upon the ENV28 peptide stimulation. Thus, at least 85% of the ENV28-specific CD8+ T cells in donor spleen cells were able to produce IFNγ.

Dynamics of Antiviral Effector Functions in the Liver

In this study, we adoptively transferred 2×10^8 immunized spleen cells containing ~10.4×10^6 ENV28-specific CD8+ T cells into 24 age-, sex-, and serum HBeAg-matched lineage 1.3.32 transgenic mice and into 24 MHC-matched nontransgenic littermates. Lineage 1.3.32 transgenic mice express all of the HBV antigens and replicate HBV in the liver and kidney (Guidotti et al., 1995). Groups of three mice were sacrificed at the indicated time points after adoptive transfer, and their intrahepatic, splenic, and peripheral blood lymphocytes were harvested for analysis of the total number of ENV28-specific CD8+ T cells present. In order to correlate the intrahepatic ENV28-specific CD8+ T cell response with downstream antiviral and immunopathological events, total liver DNA and RNA were isolated and analyzed for IFNγ and T cell messenger RNA (mRNA) expression by RNA protection assay (RPA), HBV replicative DNA content by Southern blot, HBV mRNA content by Northern blot, and evidence of liver disease by quantitating serum alanine aminotransferase (sALT) activity, as previously described (Guidotti et al., 1996).

As shown in Figure 2A, within 4 hr after transfer, ENV28-specific CD8+ T cells were already detectable in the liver (white bars) and spleen (black bars) of the transgenic recipients, and equivalent numbers of ENV28-specific CD8+ T cells were detected in the nontransgenic recipient controls (Figure S1 available in the Supplemental Data with this article online). The number of ENV28-specific CD8+ T cells in the liver of the transgenic recipients remained constant for the next 2 days, after which they expanded rapidly, reaching levels on day 7 more than 25 times higher than the 4 hr time point, after which they gradually declined (Figure 2A, white bars). Interestingly, the intrahepatic expansion of ENV28-specific CD8+ T cells appeared to have occurred at the expense of their counterparts in the spleen (Figure 2A, black bars), which were no longer detectable between days 1–5 at the same time that they were expanding in the transgenic liver and which returned to the spleen as their counterparts in the liver were contracting. These changes reflected antigen recognition, because the number of HBV ENV28-specific CD8+ T cells in the nontransgenic livers and spleens were unchanged throughout the experiment (Figure S1).

As shown in Figure 2B, the number of ENV28-specific CD8+ T cells in the liver correlated very well with the intrahepatic CD8 and CD3 mRNA content, gradually increasing by 10- to 20-fold by day 7 and then declining. In contrast, the IFNγ mRNA content rapidly peaked at 4 hr, at which time the CD3 and CD8 mRNA signals were barely detectable. Subsequently, the IFNγ mRNA content declined as the strength of the CD3 and CD8 mRNA signals increased, suggesting that the ability of ENV28-specific CD8+ T cells to produce IFNγ de-

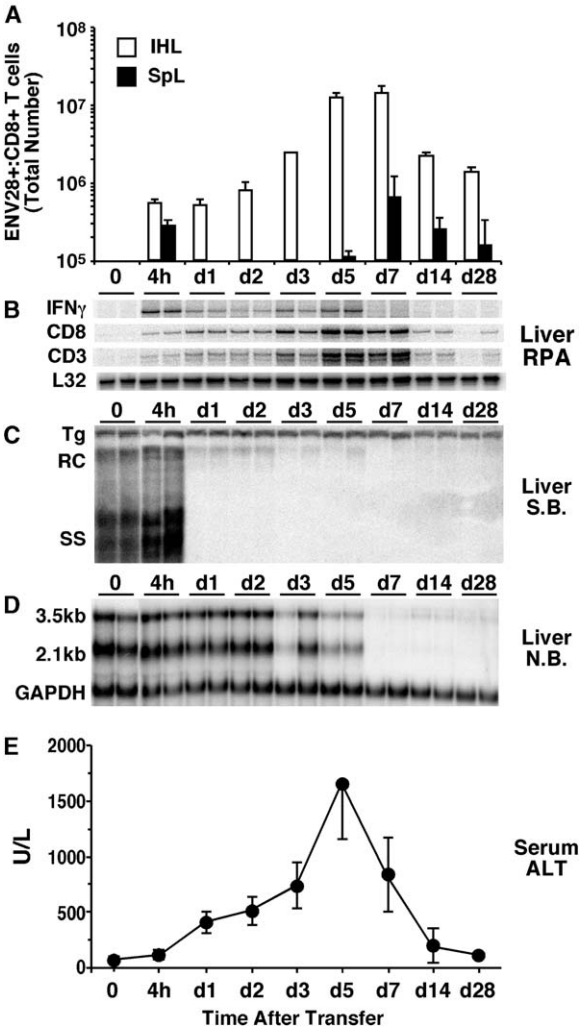

Figure 2. Kinetics of ENV28-Specific CD8+ T Cells and Their Antiviral Effector Functions in the Liver

(A) Kinetics of ENV28-specific in the liver (white) and spleen (black) of HBV transgenic mice. The data represent mean ± SD of three mice.

(B) Kinetics of T cells markers and cytokine mRNA expression in the liver. Total RNA (10 μg) isolated from liver samples was analyzed for the expression of IFNγ, CD8, CD3, and L32 by an RNase protection assay (RPA). The L32 was used to normalize the amount of RNA loaded in each lane.

(C) Effect of ENV28-specific CD8+ T cells on HBV replication in the liver. Southern blot analysis (S.B.) of 30 μg of total liver DNA isolated from the same mice. Bands corresponding to the expected size of the integrated transgene (Tg), relaxed circular (RC), and single-stranded (SS) HBV DNA were indicated. Bands corresponding to the integrated transgenes can be used to normalize the amount of DNA bound to the membrane.

(D) Effect of ENV28-specific CD8+ T cells on HBV gene expression in the liver. Northern blot analysis (N.B.) of 20 μg of total liver RNA isolated from the same mice. GAPDH was used to normalize the amount of RNA bound to the membrane.

(E) Serum alanine aminotransferase (sALT) activity in the same mice is expressed as units/liter. The data represent mean ± SD of three mice.

creased as the T cells expanded. As expected, HBV replicative intermediates almost completely disappeared from the liver as early as day 1 (Figure 2C) without a decrease in viral gene expression (Figure 2D) or a proportional increase in sALT activity (Figure 2E), suggesting that the rapid burst of IFNγ production inhibited viral replication noncytolytically and posttranscriptionally, as previously described (Guidotti et al., 1996). Note that sALT activity increased in concert with the increase in CD3 and CD8 mRNA content in the liver, suggesting that they were related events. Note also that the sALT activity peaked (Figure 2E) and viral RNA began to decline (Figure 2D) on day 5, after which the viral RNA virtually disappeared and the sALT activity declined, suggesting a relationship between these events as well. These findings were reinforced by the observations that the number of ENV28-specific CD8+ T cells (Figure 2A), the intrahepatic IFNγ, CD3, and CD8 mRNA levels (Figure 2B), and the sALT activity (Figure 2E) returned to or toward baseline once the viral RNA was undetectable, suggesting that antigen recognition was decreasing as a result of the suppression of viral gene expression (Figure 2D). The inhibition of viral RNA expression also coincided with the reappearance of ENV28-specific CD8+ T cells in the spleen (Figure 2A), suggesting that the tissue tropism or compartmentalization of the ENV28-specific CD8+ T cells is tightly regulated by the level of viral antigen synthesis in the liver.

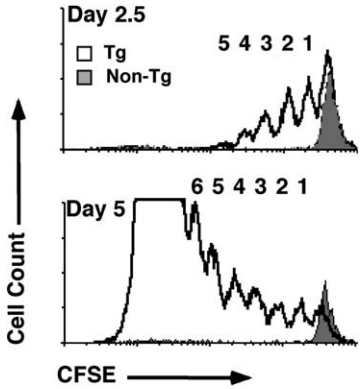

Figure 3. Rapid Cell Division of ENV28-Specific CD8+ T Cells in the Liver

2 × 10^8 of HBsAg-immunized spleen cells were labeled with 10 μM CFSE and transferred to either HBV transgenic or nontransgenic mice. The cell divisions of ENV28-specific CD8+ T cells in the liver were monitored on days 2.5 and 5 after the transfer. Histograms represent the ENV28-dimer+/CD8+ cells in HBV transgenic (white) and nontransgenic liver (gray).

The expansion in the number of intrahepatic ENV28-specific CD8+ T cells could be due to their proliferation in situ or their recruitment from the spleen. To distinguish between these alternatives, we labeled the primed spleen cells with 5,6-carboxyfluorescein diacetate succinimidyl estater (CFSE) before transferring them into transgenic and nontransgenic mice. Groups of three transgenic and nontransgenic mice were sacrificed 2.5 days and 5 days later, and the cell divisions of the ENV28-specific CD8+ T cells in the liver were monitored. As shown in Figure 3, a fraction of ENV28 specific had already divided up to five times by day 2.5 after the transfer, and they continued to divide until the majority of ENV28-specific CD8+ T cells had divided more than six times 5 days later, suggesting that the

massive expansion of intrahepatic ENV28-specific CD8+ T cells shown in Figure 2A reflects their proliferation in situ. In contrast, CFSE-labeled spleen cells did not divide when they were transferred into nontransgenic littermates (Figure 3, shaded peaks), suggesting that cell division in the transgenic recipients was triggered by antigen recognition.

Intrahepatic Antigen Recognition Triggers Phenotypic and Functional Changes in the ENV28-Specific CD8+ T Cell Populations in the Liver

The results described above suggest that the effector function of ENV28-specific CD8+ T cells rapidly evolved as they proliferated in the liver of HBV transgenic mice. To characterize that specific response, in the same animals displayed in Figure 2, we used multicolor FACS analysis to determine the extent to which the ENV28-specific CD8+ T cells in their livers and spleens coexpressed CD25, CD69, and IFNγ at various time points after adoptive transfer, with IFNγ production being examined both directly ex vivo and after in vitro stimulation by the ENV28 peptide. The results were compared with the donor spleen cells in Figure 4. To examine the impact of antigen recognition on the expression of these markers, we compared them with the steady-state content of hepatic HBV RNA, which is displayed in Figure 4A as the ratio of HBV RNA:GAPDH mRNA calculated from the Northern blot results shown in Figure 2D.

As shown in Figures 4B and 4C (white bars), the ENV28-specific CD8+ T cells in the liver of the transgenic recipients were very rapidly activated, because most of them expressed CD25+ (52%) and CD69+ (74%) at the 4 hr time point (Figure 4B and 4C). This presumably reflects antigen recognition, because neither marker was induced in the spleen of the same transgenic recipients (Figures 4B and 4C, black bars) or in the liver or the spleen of nontransgenic recipient controls (data not shown). By day 1, the fraction of ENV28-specific CD8+ T cells that expressed CD25 and CD69 cells increased further, reaching a peak of 76.9% and 88.4%, respectively, suggesting that nearly all the ENV28-specific CD8+ T cells that entered the liver had recognized antigen and were activated during the first 24 hr after adoptive transfer. Interestingly, the fraction of intrahepatic ENV28-specific CD8+ T cells that expressed CD25 started to decrease on day 2, and CD25 was no longer detectable by day 7, even though the total number of ENV28-specific T cells had expanded at least 20-fold by that time (Figure 2A). Importantly, the decline in CD25 expression mirrored the decline in HBV gene expression in the liver, strongly suggesting that CD25 expression was antigen driven. Surprisingly, the number of CD69+ T cells remained high and virtually unchanged in the liver of these mice for at least 28 days after the transfer, despite the disappearance of HBV RNA. These unexpected results suggest that, despite the cessation of antigen synthesis, the ENV28-specific CD8+ T cells were exposed to antigen throughout the experiment, implying that less antigen is required to induce CD69 than CD25.

Antigen recognition also triggered interesting changes in the effector function of the ENV28-specific CD8+ T cells in the liver of the transgenic recipients. Within 4 hr after adoptive transfer, virtually all of the intrahepatic ENV28-specific CD8+ T cells produced IFNγ both directly ex vivo (Figure 4D) and after in vitro peptide stim-

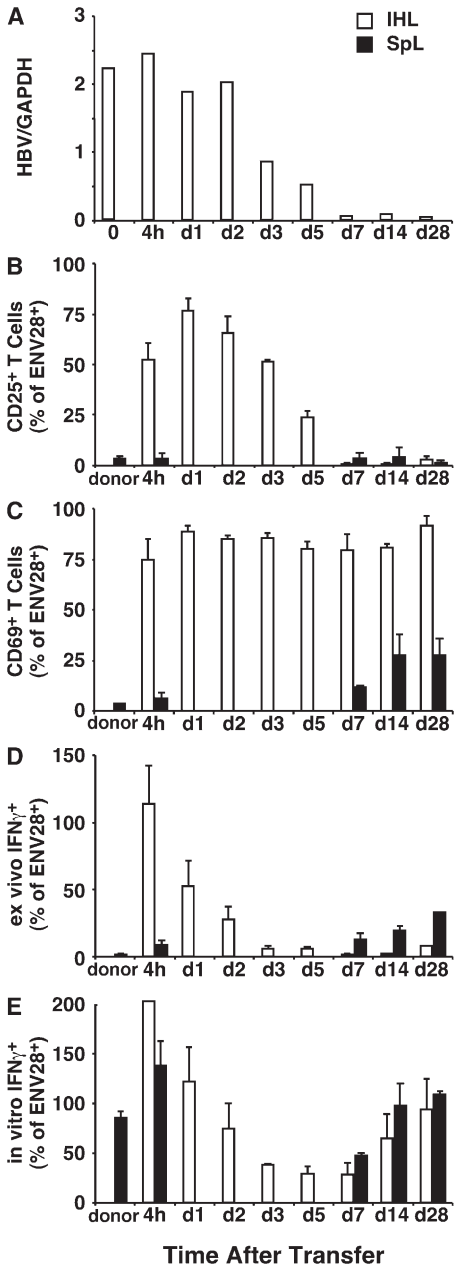

Figure 4. Phenotypical and Functional Characterization of ENV28-Specific CD8+ T Cells

The kinetics of activation marker and IFNγ expression by ENV28-specific CD8+ T cells in the liver (white) and spleen (black) were examined at indicated time points after the adoptive transfer to HBV transgenic mice.

The ratio of HBV RNA and GAPDH was calculated from the Northern blot results shown in Figure 2D (A). (B) and (C) show the kinetics of CD25 and CD69 expression on the ENV28-dimer-positive cells, respectively. (D) and (E) show the fraction of ex vivo (i.e., without peptide stimulation) and in vitro (i.e., with ENV28 peptide stimulation) IFNγ-producing CD8+ T cells in the liver, respectively. The data represent mean ± SD of three mice.

ulation (Figure 4E), similar to the induction of CD25 (Figure 4B) and CD69 (Figure 4C). Surprisingly, however, the ability of these cells to produce IFNγ decreased rapidly thereafter, becoming almost undetectable ex vivo on day 7 (Figure 4D). Importantly, the frequency of IFNγ-producing ENV28-specific CD8+ T cells returned to baseline (Figure 4E) by day 28, mirroring the cessation of HBV gene expression (Figure 4A) and contraction of the intrahepatic ENV28-specific CD8+ T cell infiltrate (Figure 2A). None of these changes occurred in the nontransgenic livers and spleens (data not shown), indicating that they reflected antigen recognition. We also monitored TNFα, IL-2, and IL-4 expression in the ENV28-specific CD8+ T cells after ENV28 peptide stimulation in vitro. ~50% of the IFNγ-producing CD8+ T cells in the donor spleen cell population expressed TNFα, but not IL-2 or IL-4, prior to transfer. After transfer, however, none of these cytokines was expressed by the intrahepatic ENV28-specific CD8+ T cells either directly ex vivo or after in vitro peptide stimulation (data not shown).

HBcAg-Specific CD8+ T Cells Are Also Compartmentalized and Impaired in Their Ability to Produce IFNγ after Antigen Recognition in the Liver

To determine whether the rapid sequestration and downregulation of IFNγ production by HBV-specific CD8+ T cells are ENV28-specific events, we transferred HBcAg-primed spleen cells containing CD8+ T cells specific for an immunodominant Kb-restricted CTL epitope located between residues 93 and 100 in the core protein (Kuhrober et al., 1997). As shown in Figure S2A, spleen cells containing ~0.25% COR93-specific, IFNγ-positive CD8+ T cells (Figure S2A) were adoptively transferred into HBV transgenic mice. As shown in Figure S2B, 5 days after transfer, ~6.7% of the total intrahepatic CD8+ T cell population was COR93 specific, whereas no COR93-specific CD8+ T cells were detected in the spleen. Importantly, like the ENV28-specific CD8+ T cell population, only 0.55% of the day 5 intrahepatic CD8+ T cells were able to produce IFNγ after peptide stimulation in vitro. These results suggest that intrahepatic sequestration and functional impairment occur after CD8+ T cells recognize antigen in the liver irrespective of the antigen specificity of the T cells.

Delayed Induction of ENV28-Specific CD8+ T Cell GrB Expression, In Vitro Cytolytic Activity, and In Vivo Pathogenic Potential

To determine whether intrahepatic antigen recognition also modulates the cytolytic activity and pathogenic potential of ENV28-specific CD8+ T cells, we monitored the intracellular content of grB and the cytolytic activity of primed donor spleen cells and intrahepatic lymphocytes before and after adoptive transfer, respectively. As shown in Figure 5A, grB expression was very low or undetectable in the donor spleen cells and in the 4 hr intrahepatic lymphocytes (IHL) population, but it increased on days 3 and 5 as the T cells expanded (Figure 2A) and as sALT activity increased in vivo (Figure 2E). This contrasted strikingly with the decrease in IFNγ production that was observed in the same time period (Figures 4D and 4E).

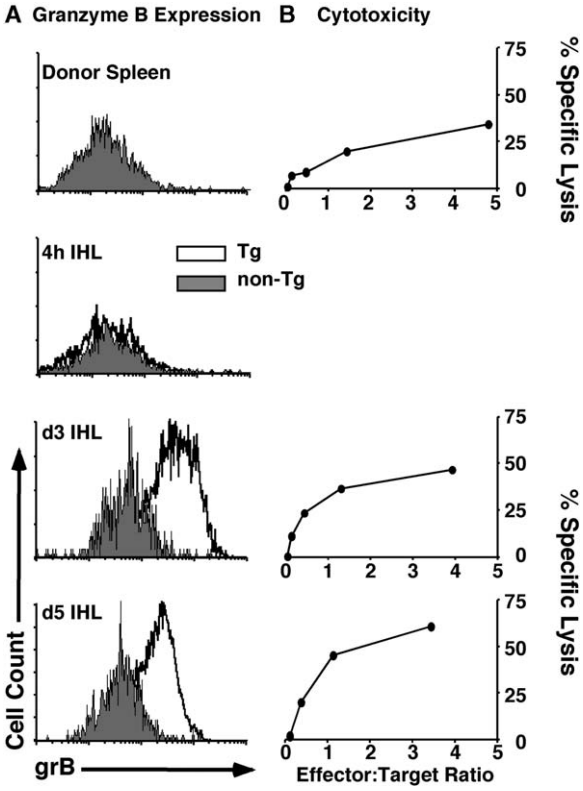

Figure 5. Delayed but Sustained Kinetics of Granzyme B Expression and In Vitro Cytolytic Activity of ENV28-Specific CD8+ T Cells

(A) Intracellular granzyme B (grB) expression of ENV28-specific CD8+ T cells in donor spleen cells and IHLs from HBV transgenic (white histograms) and nontransgenic (gray histograms) at 4 hr, day 3, and day 5 after adoptive transfer.

(B) Donor spleen cells and IHLs from HBV transgenic mice were tested for cytotoxicity in a 5 hr 51Cr release assay ex vivo. To compare cytotoxicity on a per cell basis, the effector:target (E:T) ratios were adjusted based on the frequency of ENV28-dimer-positive cells.

To determine whether the grB content in ENV28-specific CD8+ T cells reflected their cytolytic activity, varying numbers of donor spleen cells and day 3 and day 5 IHLs were incubated with P815preS1 target cells that express HBsAg or with parental P815 cells as a negative control. The effector:target (E:T) ratio was normalized based upon the frequency of ENV28-dimer-positive cells in the populations in order to compare the cytolytic activity on a per cell basis. As shown in Figure 5B, ENV28-specific CD8+ T cells in the donor spleens exhibited relatively weak cytolytic activity. In contrast, the intrahepatic ENV28-specific CD8+ T cells were highly cytolytic on day 3, and they remained so on day 5, showing good correlation between grB content and cytolytic activity. Collectively, these results demonstrate that the induction of cytolytic activity of ENV28-specific CD8+ T cells was relatively delayed but sustained in the transgenic recipients, in contrast to their ability to produce IFNγ, which was rapidly induced and then declined. The results also illustrate the existence of a close temporal relationship between these in vitro parameters (Figures 5A and 5B) and the pathogenic po-

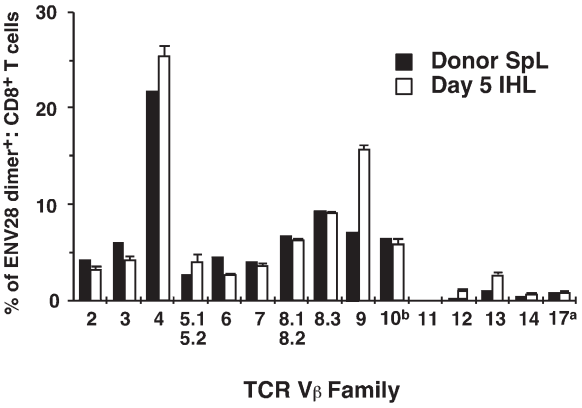

Figure 6. Analysis of TCR Vβ Usage within ENV28-Specific CD8+ T Cells

ENV28-specific CD8+ T cells in donor spleen cells (black) and day 5 IHLs from HBV transgenic mice (white) were analyzed for their TCR Vβ usage. The percentages of ENV28-specific CD8+ T cells that are recognized by each of the anti-Vβ Abs are shown. The data represent mean ± SD of three mice.

tential of the ENV28-specific CD8+ T cells in vivo (Figure 2E).

Analysis of TCR Usage

The inverse relationship of the proliferative/cytolytic and the IFNγ-producing capacities of the intrahepatic ENV28-specific CD8+ T cells after antigen recognition could reflect either the preferential clonal expansion of a highly cytopathic-IFNγ-nonproducing CD8+ T cell subpopulation or the global expansion of the original population coupled with the downregulation of their IFNγ-producing capacity. To distinguish between these alternatives, we compared the TCR usage of the ENV28-specific CD8+ T cells in donor spleen cells and IHLs isolated on day 5 after transfer by using a panel of murine Vβ chain-specific monoclonal antibodies covering 15 Vβ chain subfamilies. As shown in Figure 6, ~74% of the ENV28-specific CD8+ T cells in the donor spleen cells were detected by the panel of antibodies, reflecting the existence of polyclonal TCR repertoire. 5 days later, 85% of the ENV28-specific CD8+ T cells in the liver were detected by the antibodies, and their TCR usage was not significantly different from the donor spleen cell population. The potential expansion of a TCR subset that isn't represented in the TCR-specific monoclonal antibody panel cannot explain the increase of IFNγ-nonproducing CD8+ T cells, because the fraction of ENV28-specific CD8+ T cells detected by the antibody panel increased after expansion in the liver. Collectively, these results strongly suggest that the increase in grB production and cytolytic activity and the decrease in IFNγ production observed during the expansion phase were due to functional modulation of a globally expanding CD8+ T cell population in response to antigen recognition in the liver rather than the clonal expansion of a cytolytic but IFNγ-nonproducing subset.

ENV28-Specific Memory CD8+ T Cells Undergo Functional Oscillation Irrespective of Their Maturation Stage

To determine if the potential for functional oscillation in CD8+ T cells persisted more than 2 weeks after immunization, we adoptively transferred DNA-primed, vaccinia-boosted spleen cells harvested 4 weeks after immunization into HBV transgenic mice. The degree of coexpression of IFNγ and TNFα in the ENV28-specific CD8+ T cells was used as an index of maturation. As shown in Figure S3, the percentage of IFNγ-producing ENV28-specific CD8+ T cells that also produce TNFα in the donor peripheral blood lymphocyte population increased as a function of time after immunization from 55% at week 2 to 75.9% at week 4, indicating that maturation had occurred. In addition, these cells were CD62L low, indicating that they were effector memory rather than central memory CD8+ T cells. Spleen cells obtained from these mice on week 4 were adoptively transferred into HBV transgenic and nontransgenic recipients, and we monitored the number of ENV28-specific CD8+ T cells in the recipient livers exactly as described in Figure 2A. In addition, we examined the frequency of ENV28-specific CD8+ T cells in the hepatic and superficial (axillary and inguinal) lymph nodes to determine whether any changes that might be observed in the liver could reflect events that actually occurred in lymphocytes in the draining lymph nodes, which subsequently migrated into the liver. Finally, we determined the frequency of ENV28-specific CD8+ T cells that coexpress CD25, CD122, CD69, CD62L, IFNγ, PD-1, CTLA-4, and grB, as well as the frequency of regulatory ($CD4^+CD25^+$) T cells, at various time points after adoptive transfer to determine if functional oscillation extended to these markers as well.

The adoptively transferred ENV28-specific CD8+ T cells became activated in the liver of HBV transgenic mice (Figure 7), but not nontransgenic littermates (data not shown), similar to the results shown in Figure 4. CD69 expression (Figure 7B) was markedly increased by 4 hr after adoptive transfer and remained elevated for the duration of the experiment, suggesting persistent antigen recognition by the T cells. Antigen recognition triggered rapid induction of IFNγ in the T cells ex vivo (Figure 7C) at 4 hr after adoptive transfer. However, their ability to produce IFNγ decreased rapidly thereafter, becoming almost undetectable ex vivo on day 3 (Figure 7C), despite continued HBV gene expression at that time (Figure 7A), similar to the results shown in Figure 4D. Furthermore, the ability to produce IFNγ upon peptide stimulation followed the same kinetics. Interestingly, the rapid suppression of IFNγ production by the ENV28-specific CD8+ T cells correlated with induction of PD-1 on the T cells (Figure 7E), whereas CTLA-4 expression was never detectable on these cells, and the frequency of regulatory T cells did not change (data not shown). These results suggest that the downregulation of IFNγ production may be due to the suppressive influence of signaling via the PD-1 receptor (Freeman et al., 2000; Latchman et al., 2001). None of these changes was observed in the nontransgenic littermates (data not shown).

After these events, the intrahepatic ENV28-specific

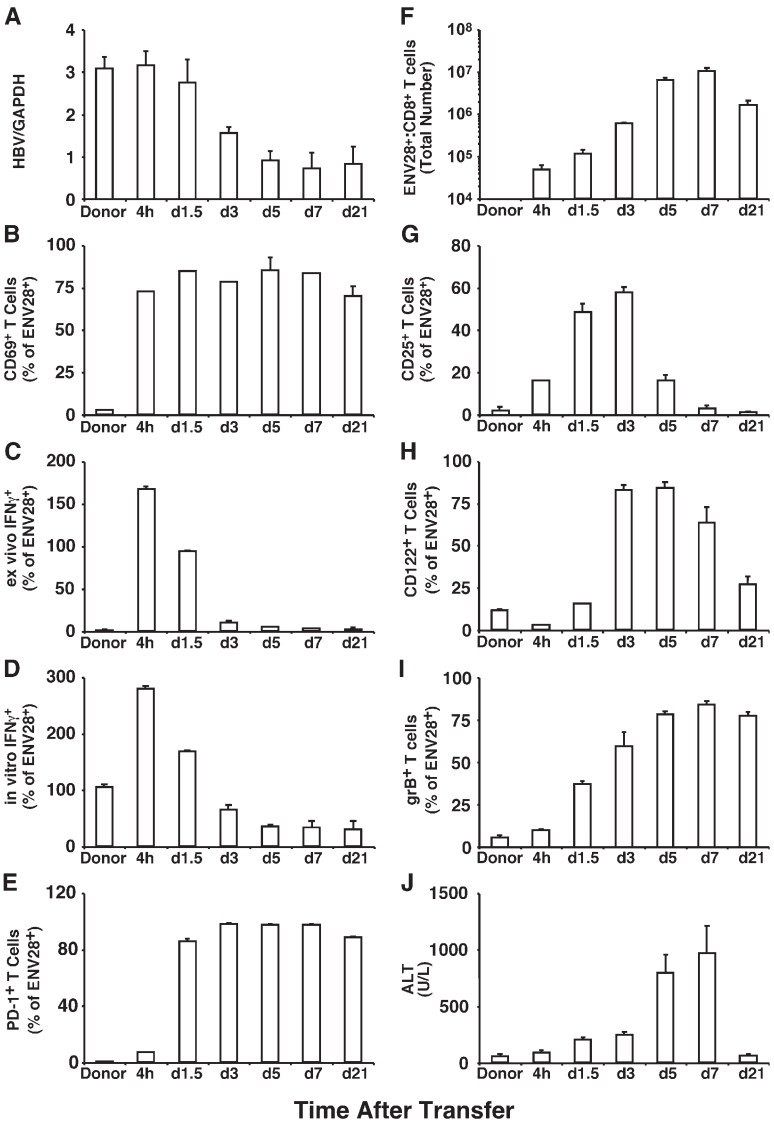

Figure 7. ENV28-Specific Memory CD8+ T Cells Also Undergo Functional Oscillation upon the Antigen Recognition in the Liver Irrespective of Their Maturation Stage

Mice were immunized by DNA-prime, vaccinia boost immunization exactly as described in Figure 1. 4 weeks later, mice were sacrificed to obtain spleen cells, and 1×10^8 of spleen cells were transferred to HBV transgenic mice or nontransgenic littermates. (A) HBV gene expression. Northern blot analysis was performed exactly as described in Figure 2, and the ratio of HBV RNA and GAPDH was shown. The fraction of ENV28-dimer-positive CD8+ T cells that coexpressed a wide variety of phenotypic and functional markers as a function of time after adoptive transfer were determined as described in the experimental procedures. These markers included CD69 (B), IFNγ either ex vivo (C) or after in vitro peptide stimulation (D), PD-1 (E), CD25 (G), CD122 (H), and grB (I). The number of intrahepatic ENV28-specific CD8+ T cells is displayed in (F), and the sALT activity in the same mice (expressed as units/liter) is shown in (J). The data represent mean ± SD of three mice.

CD8+ T cells greatly expanded by more than 20-fold on day 7, after which they declined. In contrast, the number of intrahepatic ENV28-specific CD8+ T cells was stable in the nontransgenic recipients during the entire observation period (see Figure S1B). Importantly, ENV28-specific CD8+ T cells were not detectable in the hepatic lymph nodes until day 5 (Figure S4), whereas they were detectable as early as 4 hr in the liver (Figures 2A and 7F), supporting the notion that expansion of intrahepatic ENV28-specific CD8+ T cells shown in Figure 3 reflects their proliferation in situ rather than recruitment of actively proliferating cells from the lymph nodes. As shown in Figure 7G, CD25 expression began to increase as early as 4 hr after adoptive transfer, reached a peak on day 3, and rapidly decreased to baseline level by day 7. Similar to the kinetics of T cell expansion shown in Figure 7F, CD122 (IL-2β receptor) expression (Figure 7H) was induced more slowly than CD25, reaching peak levels on days 3–5 and decreasing toward baseline thereafter. Interestingly, as IFNγ expression declined (Figures 7C and 7D), the ability of the ENV28-specific CD8+ T cells to express grB increased progressively until day 7, at which point virtually all of them were grB positive and they remained so until at least day 21. Importantly, sALT activity increased (Figure 7J) as the number of ENV28-specific CD8+ T cells (Figure 7F) that expressed grB (Figure 7I) increased in the liver, and it decreased on day 21, reflecting a 10-fold drop in the number of intrahepatic ENV28-specific CD8+ T cells (Figure 7F). Finally, the intrahepatic ENV28-specific CD8+ T cells did not express CD62L at any time after adoptive transfer (data not shown), indicating that they were not central memory T cells (Masopust et al., 2001; Sallusto et al., 1999). These results are similar to those observed after adoptive transfer of spleen cells 2 weeks after immunization, as described in Figures 2A and 4. Taken together, the data suggest that intrahepatic antigen recognition triggers functional

oscillation in the ENV28-specific CD8+ T cells irrespective of their maturation stage at the time of adoptive transfer.

Discussion

The current study was designed to address the impact of intrahepatic antigen recognition on the dynamics of a virus-specific memory CD8+ T cell response. The results revealed previously unappreciated crosstalk between the antigen-positive liver cells and the virus-specific CD8+ T cell population.

After adoptive transfer, HBV-specific CD8+ T cells behaved entirely differently in the liver and spleen of HBV transgenic mice. The intrahepatic ENV28-specific CD8+ T cells became rapidly activated to express CD25 and CD69, and they rapidly produced IFNγ (directly ex vivo), which was followed by the disappearance of HBV DNA replicative intermediates from the liver, in keeping with the known inhibitory effect of IFNγ on HBV replication (Guidotti et al., 1996; Kakimi et al., 2000). The intrahepatic ENV28-specific CD8+ T cells subsequently underwent rapid cell division and massive expansion, during which most of them converted to a CD25−/CD69+ phenotype. The rapid cell division presumably occurred in the liver, because ENV28-specific CD8+ T cells were not detectable in the hepatic lymph nodes until day 5 (Figure S4), whereas they were easily detectable in the liver as early as 4 hr after transfer (Figures 2A and 7F and Figure S4), and they were actively dividing in the liver by day 2.5 after transfer (Figure 3). As the CTLs were expanding and acquiring grB expression, serum ALT activity increased and HBV gene expression was extinguished, at which point the number of ENV28-specific CD8+ T cells began to decline. Unexpectedly, even before ENV28-specific CD8+ T cells began to expand, they lost the ability to produce IFNγ (both ex vivo and after peptide stimulation), demonstrating the rapid but transient nature of cytokine production by the ENV28-specific CD8+ T cells. In contrast, the cytolytic activity of ENV28-specific CD8+ T cells increased as they were expanding and losing the ability to produce IFNγ. Furthermore, the behavior of HBV-specific CD8+ T cells in the liver was very different from their counterparts in the spleen, because the virus is only produced in the liver and kidney of these animals, not in the spleen or other lymphoid organs. Indeed, the ENV28-specific CD8+ T cells completely disappeared from the spleen within 24 hr, and they did not reappear until day 7, at which point viral RNA was no longer detectable in the liver. We believe that the disappearance of these cells from the spleen presumably reflects their rapid sequestration in the liver rather than massive cell death in situ, because they did not show evidence of antigen recognition in the spleen (Figures 4B–4D, black bar). Interestingly, a small fraction of splenic CD8+ T cells produced IFNγ ex vivo when the ENV28-specific CD8+ T cells reappeared in the spleen, perhaps reflecting antigen presentation by dendritic cells that have processed antigen from apoptotic hepatocytes, because this occurred during and after the peak of sALT activity. Importantly, the sequestration and functional impairment was independent of the antigen specificity or maturation stage of the T cells because it was observed after transfer of both ENV28- and COR93-specific CD8+ T cells into these animals and after transfer of ENV28-specific CD8+ T cells obtained from donor mice 4 weeks after DNA-prime, vaccinia boost immunization. Collectively, these results demonstrate that antigen presentation by hepatocytes (i.e., nonprofessional antigen-presenting epithelial cells in the liver) has a profound effect on the activation state, dynamics, compartmentalization, and effector function of virus-specific CD8+ T cells in vivo.

It is noteworthy that the expression of two major activation markers, CD25 and CD69, by the intrahepatic HBV-specific CD8+ T cells displayed very different kinetics in these experiments. CD25 is required for high-affinity binding of IL-2, and its expression level is regulated by TCR signaling and IL-2 (Depper et al., 1985; Kim and Leonard, 2002; Leonard et al., 1982). CD69 expression is also regulated by TCR signaling and is generally regarded as the most sensitive marker for antigen recognition by CD8+ T cells (Castellanos et al., 1997; Testi et al., 1989). We do not understand why CD25 was downregulated in this study. Further, in view of those findings, we don't understand why CD69 was not downregulated, because both were induced by antigen recognition. It is possible that stronger antigenic stimuli are necessary to sustain CD25 expression than CD69. It is also possible that autocrine or paracrine IFNγ produced by ENV28-specific CD8+ T cells regulates CD25 expression by previously unrecognized mechanisms, as its kinetics followed the kinetics of IFNγ production with a slight delay. Because IL-2 is required for the sustained expansion of CD8+ T cells within nonlymphoid organs (D'Souza and Lefrancois, 2003; D'Souza et al., 2002), it is not surprising that the intrahepatic ENV28-specific CD8+ T cell population began to contract shortly after CD25 expression became undetectable. Nonetheless, the sustained upregulation of CD69 expression by the HBV-specific intrahepatic CD8+ T cells strongly suggests that antigen was still present at some level in the liver and that CD69 induction is more sensitive than CD25 as an indicator that low levels of antigen are present. Interestingly, expression of CD122 was induced more slowly than CD25 and appeared to be more closely associated with T cell expansion and contraction. CD122 is the β subunit shared by the IL-2 and IL-15 receptors and responsible for IL-15-dependent T cell proliferation (Judge et al., 2002; Zhang et al., 1998). The delayed kinetics of CD122 expression relative to CD25 may suggest that the signaling via CD25 is necessary for the upregulation of CD122 and that the switch from IL-2 to IL-15 responsiveness may have occurred in the expanding T cells.

The differential kinetics of noncytolytic and cytolytic effector functions of CD8+ T cells was particularly interesting. Rapid IFNγ expression and delayed emergence of cytotoxicity are characteristics of memory T cells (Slifka et al., 1999; Wolint et al., 2004). However, these studies were performed in vitro and reflected the response to peptide stimulation. The current results demonstrate that IFNγ is rapidly induced in memory CD8+ T cells in vivo, as shown by the rapid induction of IFNγ mRNA in the liver after adoptive transfer and by the ability of the corresponding intrahepatic lymphocytes

to produce IFNγ directly ex vivo, i.e., without peptide stimulation, as early as 4 hr after they entered the liver. Furthermore, we showed that the absolute number of intrahepatic CD8+ T cells was relatively low at that early time point but those cells displayed the highest level of IFNγ production at any time point examined both by intracellular cytokine staining and by RPA. Although it is theoretically possible that inflammatory cells other than ENV28-specific CD8+ T cells could have contributed to the rapid induction of IFNγ mRNA expression in vivo, we consider this unlikely for the following reasons. First, ex vivo IFNγ-producing CD8+ T cells were not observed after adoptive transfer into nontransgenic littermates, suggesting that IFNγ induction was antigen specific. Second, more than 95% of all the functional HBsAg-specific CD8+ T cells were ENV28 specific (Figure 1), making it unlikely that subdominant CD8+ T cells contributed to the IFNγ production. Third, the IFNγ-producing CD8+ T cells did not express DX5 (data not shown), ruling out the possibility that the IFNγ-producing cells were NK or NKT cells. In addition, we have previously shown that the same amount of IFNγ is produced in the liver after the adoptive transfer of ENV28-specific CD8+ T cell clones into IFNγ-deficient HBV transgenic mice and wild-type HBV transgenic mice (McClary et al., 2000), suggesting that the intrahepatic IFNγ is being produced by the adoptively transferred virus-specific CD8+ T cells and not host-derived antigen-nonspecific inflammatory cells in the liver. Taken together, the results suggest that maximum IFNγ expression in the liver is achieved very rapidly by small numbers of antigen-specific CD8+ T cells and that this inhibits viral replication very effectively even before the massive expansion of the T cells in the liver.

Perhaps the most unexpected result in this study was the early loss of the ability of the HBV-specific CD8+ T cells to produce IFNγ in the liver. We suggest that this reflects the functional impairment of ENV28-specific CD8+ T cells by sustained antigen stimulation rather than by the loss of antigenic stimulation, because the intrahepatic HBV mRNA level did not decrease until 3 days or more after adoptive transfer, yet the IFNγ-producing population had decreased by ~90% during this interval. In contrast, the in vitro cytolytic activity and the cytopathic effect of the ENV28-specific CD8+ T cells emerged much later than their IFNγ-producing capacity; indeed, they appeared as IFNγ levels were waning. Importantly, the rapid loss of IFNγ-producing capacity of the ENV28-specific CD8+ T cells and the delayed induction of their cytolytic activity were not due to the preferential expansion of a highly cytolytic IFNγ nonproducing CD8+ T cell population, because the TCR usage of the ENV28-specific CD8+ T cells was not significantly different before and after transfer. This is consistent with the results reported by Fuller et al., in which CD8+ T cell exhaustion occurred without changes in TCR usage during chronic LCMV infection (Fuller et al., 2004). Furthermore, the rapid induction and decline in IFNγ production and delayed emergence of cytolytic activity were similarly observed when matured memory T cells (i.e., 1 month after DNA-prime, vaccinia boost immunization) were adoptively transferred to HBV transgenic mice (Figure 7), suggesting that the intrahepatic antigen recognition triggers functional oscillation in T cells irrespective of their maturation stage at the time of adoptive transfer. Collectively, we suggest that the rapid loss of IFNγ-producing capacity of ENV28-specific CD8+ T cells and the delayed induction of their cytolytic activity reflect the functional modification of the entire ENV28-specific T cell population.

Interestingly, the loss of IFNγ expression coincided with the strong induction of PD-1 on ENV28-specific CD8+ T cells (Figure 7E). In contrast, CTLA-4 expression was never detectable on the HBV-specific T cells, and no changes were observed in the frequency of intrahepatic regulatory (CD4+CD25+) T cells at any time point after adoptive transfer (data not shown). PD-1 plays an important role in maintaining tolerance to autoantigens and tumors (Dong et al., 2002; Iwai et al., 2002; Okazaki et al., 2003). Engagement of TCR and PD-1 modulates TCR signaling by activating the tyrosine phosphatase SHP-2 (Latchman et al., 2001). We therefore suggest that the downregulation of IFNγ production by CD8+ T cells after antigen recognition in the liver may be due to suppressive signaling via the upregulated PD-1 receptor. Experiments are currently underway to test this hypothesis.

The importance of these results would be heightened if they also occur during natural infections. Analysis of the relative kinetics of cytokine production and cytolytic activity during infection is difficult because virus-specific CD8+ T cells are relatively infrequent during a primary CD8+ T cells response. It is worth mentioning, however, that serum IFNγ levels are induced before the expansion of virus-specific CD8+ T cells during primary LCMV infection (Pien et al., 2002). Furthermore, functional impairment of CD8+ T cells has been extensively studied in chronic LCMV infection. In contrast to our study, however, the cytolytic activity of LCMV-specific CD8+ T cells was more easily impaired than their IFNγ-producing ability (Wherry et al., 2003a; Zhou et al., 2004). We do not understand the basis for the difference, but it may reflect the nature of the target organs. In our system, antigen recognition by HBV-specific CD8+ T cells occurs almost exclusively in the liver, where the antigen-expressing cells are not professional antigen-presenting cells (APCs) and they are tightly packed, whereas antigen recognition by LCMV-specific CD8+ T cells occurs systematically in many cell types, including dendritic cells and other professional APCs. Further studies are warranted to examine the basis for these differences. Interestingly, IFNγ gene expression in the liver correlates with viral clearance and precedes the onset of liver disease in acutely HBV-infected chimpanzees (Chisari, 2000; Guidotti et al., 1999; Wieland et al., 2004), and none of these events occurred when CD8+ T cells were depleted at the peak of HBV infection (Thimme et al., 2003). Thus, it is possible that sequential activation of noncytolytic and cytolytic CD8 T cell effector functions occurs during HBV infection, LCMV infection, and other viral infections as well.

In summary, the results described herein illustrate the power of the HBV-positive liver to activate and sequester HBV-specific CD8+ T cells in the liver, the prodigious proliferative capacity of HBV-specific CD8+ T cells within the liver, and the sequential and reciprocal nature of the relationship between the IFNγ-producing and cytolytic potential of those T cells. The data suggest that

sequential stimulatory and inhibitory effects of antigen recognition on IFNγ production and cytolytic activity could easily produce an oscillating cycle on which antiviral effector functions are alternately triggered and suppressed by a fluctuating antigen burden. We are attracted by the hypothesis that these events could limit the amount of tissue injury in the context of a vigorous T cell response, and they could explain why IFNγ-nonproducing but cytolytic T cells are commonly present during persistent viral infections of the liver (Lechner et al., 2000; Thimme et al., 2001).

Experimental Procedures

Mice

HBV transgenic mouse lineage 1.3.32 (inbred C57BL/6, H-2^b) has been previously described (Guidotti et al., 1995). These animals express all of the HBV antigens and replicate HBV in the liver and kidney at high levels without any evidence of cytopathology. Lineage 1.3.32 and C57BL/6 mice (H-2b) were bred for one generation against B10.D2 mice (H-2^d) to produce F1 hybrids (designated B6D2). In all experiments, the mice were matched for age (8 weeks), sex (male), and HBeAg levels in their serum before experimental manipulations.

Peptide, Plasmid, and Recombinant Vaccinia Virus

Peptide, plasmid, and recombinant vaccinia viruses used in this study have been previously described (Ando et al., 1993; Ishikawa et al., 1998; Kuhrober et al., 1997; Schirmbeck et al., 2003; Sette et al., 2001). Details are provided in the Supplemental Experimental Procedures.

Immunization of Mice and Adoptive Transfer

B6D2 mice were immunized by using a plasmid DNA prime-vaccinia virus boost regimen exactly as described (Kakimi et al., 2002). 14 or 28 days after the booster immunization, mice were sacrificed, and spleen cells were injected intravenously into syngenic transgenic and nontransgenic recipients. Groups of three mice were sacrificed at various time points after transfer and their livers, lymph nodes, peripheral blood, and spleen were harvested for further analysis. Detailed procedures can be found in the Supplemental Experimental Procedures.

CFSE Labeling

Cells were labeled with CFSE (Molecular Probes) as previously described (Kakimi et al., 2002).

Immunofluorescent Staining and FACS Analysis

Recombinant soluble dimeric H-$2L^d$:Ig and H-$2K^b$:Ig Fusion Protein (BD/PharMingen) complexed with HBV ENV28 peptide (ENV28-dimer) and COR93 peptide (COR93-dimer) were prepared according to the manufacturer's instructions. Anti-mouse CD4, -CD8, -CD3, -CD25, -CD69, -CD62L, -CD122, PD-1, -CTLA-4, -TCRs, IFNγ, -TNFα, -IL-2, and IL-4 (all purchased from BD/Pharmingen) and anti-human granzyme B (Caltag) were used as described in the Supplemental Experimental Procedures. Cells were acquired by using either a FACSCalibur flow cytometer (BD Bioscience) or Digital LSRII flow cytometer (BD Bioscience), and data were analyzed by using CELLQuest software (BD Bioscience) or FlowJo (Tree Star Inc.).

Cytotoxicity Assays

^{51}Chromium (Cr) release assays were performed directly ex vivo by using 5 × 10^3 ^{51}Cr-labeled P815preS1 target cells that express HBsAg and control parental P815 cells as described (Ando et al., 1993).

Tissue DNA and RNA Analyses

Total liver DNA and RNA were analyzed for HBV replicative intermediates by Southern blot, for HBV RNA by Northern blot, and for cytokine transcripts by RNase protection assay exactly as previously described (Guidotti et al., 1995, 1996). The relative abundance of specific DNA and RNA molecules was determined by phosphor imaging analysis, using the Optiquant image analysis software (Packard).

Biochemical Analyses

The extent of hepatocellular injury was monitored by measuring sALT activity at multiple time points after treatment as previously described (Guidotti et al., 1995; Moriyama et al., 1990).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and four figures and are available with this article online at http://www.immunity.com/cgi/content/full/23/1/53/DC1/.

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